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Further evidence of the southern Mediterranean medicinal leech *Hirudo verbana* (Annelida, Hirudinea) feeding on fish, with a review of the use of fish hosts by *Hirudo* spp.

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ABSTRACT

Further evidence of the southern Mediterranean medicinal leech *Hirudo verbana* (Annelida, Hirudinea) feeding on fish, with a review of the use of fish hosts by *Hirudo* spp.

Although often considered primarily hematophagous on mammalian blood, the leeches belonging to the genus *Hirudo* can feed on several other vertebrate hosts. However, to date, little is known about the feeding ecology of medicinal leeches in the wild, and the few available observations mostly deals with the European medicinal leech, *Hirudo medicinalis*. Since even closely related *Hirudo* species have different functional morphology and ecological preferences, the observations carried out on one species cannot be automatically generalized to the other medicinal leeches, and targeted surveys must be realised to better understand the feeding habits of these leeches. A wide geographical belt ranging from the Iberian Peninsula to the Caucasus and Central Asia is inhabited by the southern European medicinal leech, *Hirudo verbana*. Although quantitative data are missing, the species seems characterized by a general decline throughout its distribution range, whose causes are not fully understood. Accordingly, a better understanding of *H. verbana* dietary composition is crucial to characterise its role in the trophic webs of the ecosystems it inhabits, with important consequences for the correct management of the populations of the species. Here, we report the observation of *Hirudo verbana* feeding on fish in the wild in southern Italy. This observation constitutes one of the first direct evidences of the species using fish as host organisms, since to date wild *H. verbana* had been observed mostly feeding on amphibians and mammals. Such information is useful to better characterise the diet of the wild populations of *H. verbana*, and to understand the trophic resources which could be exploited by the species in different ecosystems. Finally, a review of all the available evidence dealing with the use of fish as hosts organisms by *Hirudo* spp. is carried out.

KEY WORDS: feeding ecology, Hirudinidae, mtDNA COI, *Hirudo* conservation, hirudiniasis.

RESUMEN

Nuevas pruebas de que la sanguijuela medicinal del Mediterráneo meridional* Hirudo verbana *(Annelida, Hirudinea) se alimenta de peces, con una revisión del uso de peces huéspedes por* Hirudo *spp.

Aunque a menudo se las considera principalmente hematófagas de sangre de mamíferos, las sanguijuelas del género Hirudo *pueden alimentarse de otros huéspedes vertebrados. Sin embargo, hasta la fecha se sabe poco sobre la ecología alimentaria de las sanguijuelas medicinales en libertad, y las pocas observaciones disponibles se refieren sobre todo a la sanguijuela medicinal europea,* Hirudo medicinalis*. Dado que incluso las especies de* Hirudo *estrechamente emparentadas tienen diferente morfología funcional y preferencias ecológicas, las observaciones realizadas sobre una especie no pueden generalizarse automáticamente a las demás sanguijuelas medicinales, y deben realizarse estudios específicos para comprender mejor los hábitos alimentarios de estas sanguijuelas. La sanguijuela medicinal del sur de Europa,* Hirudo verbana*, habita un amplio cinturón geográfico que va desde la Península Ibérica hasta el Cáucaso y Asia Central. Aunque faltan datos cuantitativos, la especie parece caracterizarse por un declive general a lo largo de su área de distribución, cuyas causas no se comprenden del todo hasta la fecha, por lo tanto, es crucial conocer mejor la composición de la dieta de* H. verbana *para caracterizar su papel en las redes tróficas de los ecosistemas que habita, con importantes consecuencias para la correcta gestión de las poblaciones de la especie. Aquí reportamos la observación de* Hirudo verbana *alimentándose de peces en estado salvaje en el sur de Italia. Esta observación constituye una de las primeras pruebas directas de que la especie utiliza peces como organismos hospedadores, ya que hasta la fecha* H. verbana *silvestre se había observado principalmente alimentándose de anfibios y mamíferos. Esta información es útil para caracterizar mejor la dieta de las poblaciones silvestres de* H. verbana *y para comprender los recursos tróficos que podría explotar la especie en diferentes ecosistemas. Por último, se lleva a cabo una revisión de todas las pruebas disponibles sobre el uso de peces como organismos hospedadores por parte de* Hirudo *spp.*

PALABRAS CLAVE: *ecología alimentaria, Hirudinidae, mtDNA COI, conservación de* Hirudo*, hirudiniasis.*

INTRODUCTION

In the last decades, morphological, karyological, and genetic evidence consistently showed that the diversity of the leech genus *Hirudo* was largely underestimated, and that different *Hirudo* species were lumped under the binomen *Hirudo medicinalis* (e.g., Utevsky et al., 2009, 2010; Darabi-Darestani et al., 2018; Wang et al., 2022). According to current taxonomy, seven species are ascribed to this genus, with three of them occurring in Europe with autochthonous populations (Trontelj & Utevsky, 2012). Albeit data about the ecological preferences and requirements are currently lacking for some of the taxa, an increasing amount of evidence shows that the different species are characterized by different ecology, as also supported by differences in their salivary gland secretion (Baskova et al., 2008), mouth parts morphology (Kovalenko & Utevsky, 2015), hosted symbiotic microorganisms (Laufer et al., 2008), and phenology (Kovalenko & Utevsky, 2012). Accordingly, Utevsky et al. (2010) suggested that the parapatric distribution areas of *Hirudo* species are mostly determined by their different ecological preferences, although recent evidence suggests an important role of Pleistocene glacial cycles as well in shaping the current diversity pattern of some taxa (Trontelj & Utevsky, 2012; Arias et al., 2021).

Although often considered by the popular belief to be strictly hematophagous on mammals, hirudinid leeches have plastic feeding habits, which allow them to cope with different environmental conditions and prey hosts. Available data mostly refer to the central and northern European *Hirudo medicinalis* Linnaeus, 1758 and attest that most of its diet is based on amphibians and birds, with only occasional blood meals on mammals and fish (Johnson, 1816; Wilkin & Scofield, 1990; Elliott, 2008, Kutschera & Elliott, 2014 and references therein). Only scarce information is currently available for other *Hirudo* species: *Hirudo troctina* Johnson, 1816 has been reported to feed on fish (Johnson, 1816) and amphibians (Merabet & Karar, 2021, and references therein), and *Hirudo orientalis* Utevsky & Trontelj, 2005 has been observed feeding on *Carassius auratus* in captivity (Khiabanian & Salimi, 2017, sub "*Hirudo medicinalis*"). *Hirudo verbana* Carena, 1820 largely uses amphibian (Marrone et al., 2021, and references therein) and mammal (Stschegolev, 1938, sub "*Hirudo medicinalis*") hosts for feeding and was observed to show a cannibalistic behaviour in captivity (Ceylan & Erbatur, 2012); furthermore, based on the molecular genomic metabar-

coding of bloodmeals of trafficked *H. verbana* individuals confiscated in Canada, Williams et al. (2020) collected evidence of *H. verbana* feeding on some fish species, a finding also corroborated by recent observations carried out in Turkey by Ceylan et al. (2023).

The paucity of information about the diet of most of the currently known *Hirudo* species prevent their sound long-term management, which is particularly important in the light of the threatened status of most *Hirudo* species and populations throughout their distribution range (Utevsky et al., 2010): different *Hirudo* species are listed in Annex V of the EU "Habitats Directive", Annex II of the "CITES convention", and Appendix III of the "Bern Convention". Here we report on an occasional observation of an adult *Hirudo verbana* individual feeding on a grey mullet in a river in southern Italy, which is one of the first corroborated evidences of a predatory behaviour of the species on fish in the wild.

METHODS

A juvenile mugilid fish belonging to the genus *Chelon* Artedi, 1793 with an attacked hirudinid leech was observed by DB on 30/09/2023 in the river Idro (province of Lecce, Apulia, southern Italy), about 1 km from the river mouth (WGS84 geographical coordinates: 40.142854 N, 18.478017 E). The river Idro originates by the resurgence of karstic springs close to the coastline and, after a relatively short course of about 3 km, it flows into the port of the town of Otranto. It is formed by the confluence of two branches, the main one fed by the spring of Carlo Magno, and the secondary one, also called Canale Bollato, which is fed by the San Giuseppe spring. The entire river underwent a major regulation work in the 20th century, which transformed the riverbed in an artificial channel. The chemical values of the water measured at the two main springs showed moderately alkaline pH values, a temperature of 17.2-17.7 C° and electric conductivity ranging from 800 to 930 µS/cm (Bonfrate et al., 2002).

At the site of the finding, situated along the secondary branch of the river (Canale Bollato), the watercourse is characterized by bottom and banks made of concrete, with a water depth of about 25 cm and a width that goes from 40 cm to 2 meters in the final segment of its path (Fig. 1a). The water is clear and subject to a slight flow, and the riverbed is covered by various aquatic macrophytes such as *Groenlandia densa* (L.) Fourr., *Nasturtium officinale* (W. T. Aiton, 1812) and *Helosciadium nodiflorum* (L.) W. D. J. Koch (Fig. 1).

The hirudinid leech was feeding on juvenile grey mullet (Mugilidae), which seemed to have recently died. It was not possible to establish whether the leech attacked the fish when it was still alive, causing its death, or if it was already dead or moribund. However, it is well known that *Hirudo* spp. attacks on fishes often cause the death of small individuals (e.g., Blair, 1927; Khiabanian & Salimi, 2017). The fish was photographed (Fig. 1c) and then released in the river; conversely, the hirudinid leech was collected and preserved in situ in 95% ethanol.

The mugilid fish was identified based on Kottelat & Freyhof (2007) and Fortini (2016). The hirudinid leech was identified based on morphology according to Utevsky & Trontelj (2005) and Saglam et al. (2016). In addition, in the light of the existence of significant cryptic diversity and genetic structuring within *Hirudo verbana* (Arias et al., 2021), total DNA was extracted by a small piece of the caudal sucker of the collected specimen and a fragment of the mitochondrial DNA COI marker was amplified and sequenced following Kvist et al. (2022). The obtained COI sequence was then aligned with selected *Hirudo* spp. sequences downloaded from GenBank (see figure 2 for their Accession Numbers, A. N.), and the inference about the phylogenetic relationships among the taxa, based on maximum likelihood analyses (ML) and Bayesian inference of phylogeny (BI), was performed following the protocol described by Vecchioni et al. (2019).

The ethanol-preserved hirudinid leech was measured with a calliper and then deposited in the collection of Annelida of the Museo La Specola, Firenze, Italy (catalogue number: MZUF AN/6928).

In order to collect the available evidence dealing with *Hirudo* spp. feeding on fish, a review of all the available information was carried out, both through a careful bibliographical review and

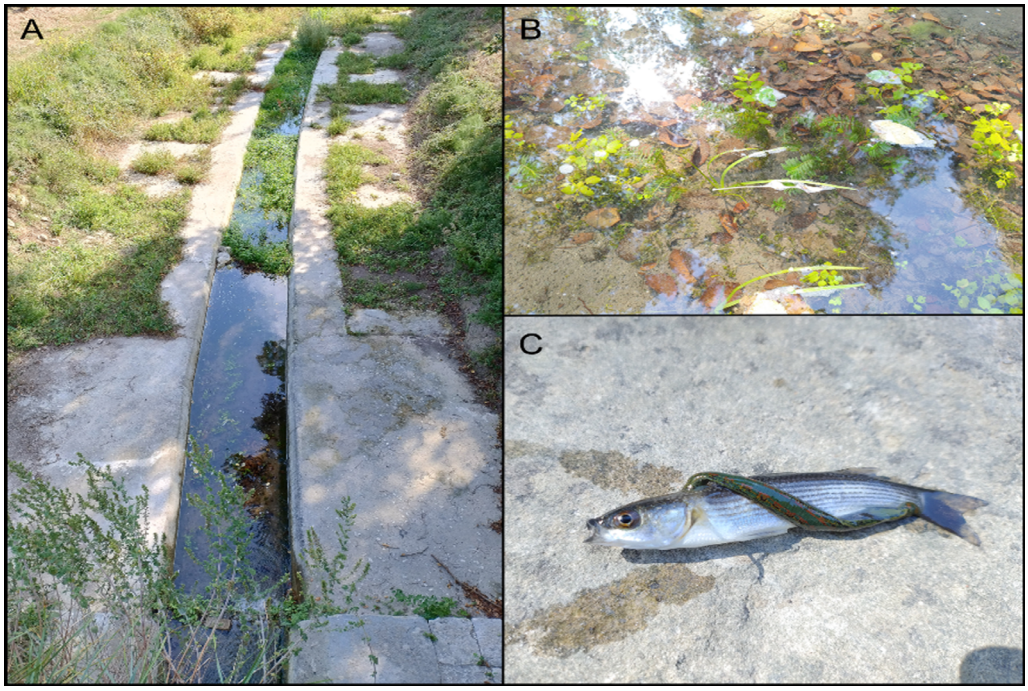

Figure 1. (a), (b) Habitat of *Hirudo verbana* at the site of Canale Bollato, River Idro; (c) *Hirudo verbana* individual feeding on a juvenile *Chelon* cf. *labrosus*. *(a), (b) Hábitat de* Hirudo verbana *en el sitio Canale Bollato, río Idro; (c) individuo de* Hirudo verbana *alimentándose de un juvenil de* Chelon *cf.* labrosus.

through interviews with other researchers and consultation of social networks.

RESULTS

Based on the morphological characters visible in photo and the habitat type (Fig. 1), the mugilid fish preyed upon by the hirudinid leech can be identified as a member of the genus *Chelon*, because of the shortness of the pectoral fins and the absence of adipose tissue covering most of the eye. Despite the difficulty to verify some characters, due to the quality of the picture and the juvenile stage of the individual, the moderate thickness on the upper lip, the absence of a dark spot at pectoral base and the absence of yellow spots at the operculum allow to tentatively identify it as *Chelon* cf. *labrosus* Risso, 1827. This is a common species along the Italian coasts and is known to be able to move upstream until freshwater sections of the rivers for feeding reasons (Fortini, 2016).

The preserved *Hirudo* specimen measured 57.5 mm, but it should be stressed that its body was contracted since a step with relaxation fluid was not implemented before fixing it in 95% ethanol (cf. Nesemann & Neubert, 1999). The length of the specimen when relaxed is thus expected to be higher than the value recorded here.

Obtained mtDNA COI sequence was uploaded to the public database GenBank (A.N. PP430157). Upon alignment, the analysis of the 644 bp-long mtDNA COI dataset produced BI and ML trees with a consistent topology, both clustering the novel *Hirudo* sequence with published Italian and Slovenian haplotypes belonging to the "western phylogroup" of *Hirudo verbana* as defined by Trontelj & Utevsky (2012) and Utevsky & Trontelj (2016). In accordance with the colour pattern (Fig. 1c), the phylogenetic trees thus consistently allow to identify the leech collected in the river Idro as *Hirudo verbana* (Fig. 2).

Overall, seven papers including primary data dealing with *Hirudo* spp. attacks on fish were retrieved, to which we here add the report of an unpublished event observed in Ukraine, and present work (Table 1).

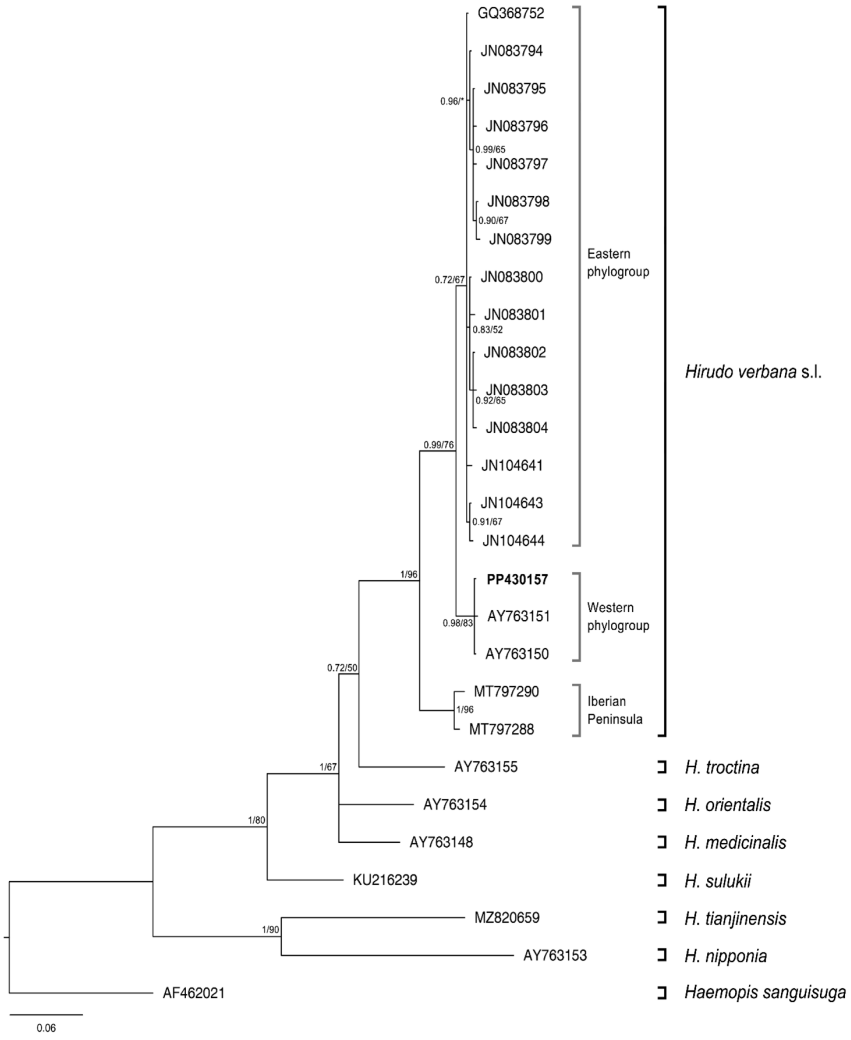

Figure 2. Bayesian phylogram of published and collected *Hirudo* specimens based on a fragment of 644 bp-long of the mtDNA COI dataset. *Haemopis sanguisuga* (Linnaeus, 1758) was used as an outgroup to root the tree (A.N. AF462021). Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (maximum likelihood, ML). Asterisks show support values lower than 50. The novel sequence is reported in bold. *Filograma bayesiano de especímenes de* Hirudo *publicados y recolectados basado en un fragmento de 644 pb de longitud del conjunto de datos de ADNmt COI.* Haemopis sanguisuga *(Linnaeus, 1758) se utilizó como grupo externo "outgroup" para enraizar el árbol (A.N. AF462021). El soporte estadístico de los nodos se presenta como probabilidades posteriores de los nodos (inferencia bayesiana de la filogenia, BI)/valores bootstrap (máxima verosimilitud, ML). Los asteriscos indican valores de apoyo inferiores a 50. La nueva secuencia aparece en negrita.*

DISCUSSION

The southern European medicinal leech, *Hirudo verbana* inhabits a wide geographical belt ranging from the Iberian Peninsula to the Caucasus and Central Asia, through the European Mediterranean countries and Asia Minor. Although quantitative data are missing, the species seems characterized by a general decline thorough its distribution range, whose causes are to date not fully understood, but unsustainable collection, habitat alteration and regression of host organisms are often considered of primary importance for the decline of *Hirudo* spp. (e.g., Davies & McLoughlin, 1996; Utevsky et al., 2010; Kutschera & Elliot, 2014).

Table 1. Synopsis of the available evidence of *Hirudo* spp. feeding on fish. Sources 1: Blair (1927); 2: Wilkin (1987); 3: Wilkin & Scofield (1990); 4: Johnson (1816); 5: Khiabanian & Salimi (2017); 6: S. Utevsky, unpublished data; 7: Williams et al. (2020); 8: Ceylan et al. (2023); 9: Present work. *: These genera were indistinguishable based on available COI sequences. *Sinopsis de las pruebas disponibles de que* Hirudo *spp. se alimenta de peces. Fuentes 1: Blair (1927); 2: Wilkin (1987); 3: Wilkin & Scofield (1990); 4: Johnson (1816); 5: Khiabanian & Salimi (2017); 6: S. Utevsky, datos inéditos; 7: Williams et al. (2020); 8: Ceylan et al. (2023); 9: presente trabajo. *: Estos géneros eran indistinguibles sobre la base de las secuencias de COI disponibles.*

Taxon	Fish host	Source	Notes
H. medicinalis	"fish"	1	
H. medicinalis	*Scardinius erythrophthalmus* (Cyprinidae)	2	
H. medicinalis	*Gasterosteus aculeatus* (Gasterosteidae)	2	
H. medicinalis	"fish"	3	Based on serological analyses
H. medicinalis	*Salmo trutta* (Salmonidae)	4	Reported sub "*Salmo Eriox*"
H. troctina	*Salmo trutta* (Salmonidae)	4	Reported sub "*Salmo Eriox*"
H. orientalis	*Carassius auratus* (Cyprinidae)	5	Reported sub *H. medicinalis*
Hirudo sp.	*Carassius* sp.	6	*H. medicinalis* or *H. verbana*
H. verbana	*Esox lucius* (Esocidae)	7	Based on iDNA
H. verbana	*Perccottus glenii* (Odontobutidae)	7	Based on iDNA
H. verbana	*Pseudorasbora parva* (Cyprinidae)	7	Based on iDNA
H. verbana	*Rutilus rutilus* (Cyprinidae)	7	Based on iDNA
H. verbana	*Cyprinus* sp. or *Carassius* sp.*	7	Based on iDNA
H. verbana	*Carassius gibelio* (Cyprinidae)	8	
H. verbana	*Gambusia holbrooki* (Poecilidae)	8	
H. verbana	*Chelon* cf. *labrosus* (Mugilidae)	9	

In Italy, *Hirudo verbana* is reported to occur throughout the mainland and its main islands, although the vast majority of the validated, recent records pertains to southern Italian peninsula and Sicily (Marrone & Canale, 2019; Marrone et al., 2021). The only other Italian *Hirudo verbana* specimen for which molecular data are available originated from Apulia as well (Trontelj & Utevsky, 2005) and, in good accordance with our results, could be ascribed to the "western clade" of the species, which is known from the western Balkan and Italian peninsulas (Utevsky & Trontelj, 2016). In addition, lacking recent data and samples, the possible occurrence of other *Hirudo* species in Sardinia and northern Italy cannot be at present ruled out since the identity of some *Hirudo* populations historically ascribed to *H. medicinalis* and *H. troctina* could not be verified. In Apulia, *H. verbana* was already reported to occur in the Idro River (Ferreri, 1995), which is thus confirmed as sustaining a population of this species. Other three *H. verbana* populations are to date reported for southern Apulia (Dequal, 1916; Ferreri, 1995; Marrone et al., 2021), and further monitoring in the area is desirable to assess its status and distribution in what seems to be a possible stronghold of this declining species. *Hirudo verbana* is not the only leech able to prey on fish in Apulian inland waters, since also the non-native piscicolid *Myzobdella lugubris* Leidy, 1851 was recently reported for Le Cesine, about 25 km from the study site (Liuzzo et al., 2018).

It is nowadays generally accepted that fish fall within the diet of hirudinid leeches (e.g., Elliott, 2008; Kutschera & Elliot, 2014), however, direct evidence of this is scarce, and nearly completely limited to *Hirudo medicinalis*. According to Kutschera & Elliott (2014), juvenile *H. medicinalis* are unable to pierce mammal skin because their jaws are not strong enough; this way, the use of amphibians or fish as prey would be mandatory at this early life stage. This is in accordance with the description presented by Johnson (1816), according to which leeches in medical use (e.g., *H. medicinalis* and *H. troctina*) subsist by feeding on fish (*Salmo trutta* Linnaeus,

1758) and frogs. Similarly, Blair (1927), report that the young British *Hirudo medicinalis* mainly feed on frogs, tadpoles, and unspecified small fish. However, Blair (1927) also observed a carp (C*yprinus carpio* Linnaeus, 1758) eating a brood of *H. medicinalis* in aquarium, thus showing the existence of complex trophic relationship between fish and leeches. Wilkin (1989) reported *H. medicinalis* feeding on the cyprinid *Scardinius erythrophthalmus* (Linnaeus, 1758) and the gasterosteid *Gasterosteus aculeatus* Linnaeus, 1758 in the wild in Britain, but did not provide information about the age or size of the *H. medicinalis* individuals observed on these fish. One of the authors of present work (SU) observed an adult *Hirudo* sp. leech attaching juvenile *Carassius* sp. at the end of the nineties in a temporary water body within Horila Dolyna (=Burned Valley) in Ukraine (WGS84 geographical coordinates: 49.624074 N, 36.543586 E). Both *Hirudo verbana* and *H. medicinalis* cohabit in that water body (Kovalenko & Utevsky, 2012) and no pictures were taken at that date, so that it is now impossible to ascertain whether they belonged to *H. verbana*, *H. medicinalis*, or both. Khiabanian & Salimi (2017) observed an attack of *H. orientalis* on the cyprinid *Carassius auratus* (Linnaeus, 1758) in aquarium, but did not collect any evidence of a similar behaviour in the wild. Finally, Ceylan et al. (2023) observed *Hirudo verbana* individuals feeding on the cyprinid *Carassius gibelio* (Bloch, 1782) and the poeciliid *Gambusia holbrooki* Girard, 1859 caught in fyke nets in Lake Eğirdir (Turkey).

Considering all the records of medicinal leeches feeding on fish, it is important to emphasize the differences in the feeding behaviour between medicinal leeches (Hirudinidae) and fish leeches (Piscicolidae). Piscicolids are well-documented ectoparasites of various fish species in both marine and freshwater environments (Sawyer, 1986; Utevsky & Trontelj, 2004). Fish leeches remain on their host for extended periods, often preferring the gill and mouth cavities of fish. While they can weaken their host and cause various kinds of damage, piscicolids typically do not kill their host. Additionally, piscicolid leeches are usually much smaller than their fish hosts, which serve as habitats for these ectoparasites (Sawyer, 1986; Utevsky & Trontelj, 2004; Utevsky, 1997; Utevsky et al., 2021). These factors collectively suggest that piscicolids are typical parasites. In contrast, medicinal leeches exhibit a very different feeding strategy. They attach to relatively small fish, do not use the body surface of their fish hosts as a habitat, and do not remain on fish for days or weeks (Ceylan et al., 2023; our observations). It can be thus hypothesized that medicinal leeches kill their prey, indicating that hirudinids should be considered predators hunting fish, rather than parasites. Further studies should be desirably carried out to better test this hypothesis, especially since the reduction of usual *Hirudo* spp. habitats and hosts, due to the global decline of amphibians (Beebee & Griffiths, 2005; Cushman, 2006) and the ongoing disappearance of smaller water bodies, might facilitate the interactions between medicinal leeches and fish.

Based on the number and size of denticles on jaws, Kovalenko & Utevsky (2015) hypothesized that, whereas *Hirudo orientalis*, *H. medicinalis* and *H. troctina* mainly feed on amphibians, *H. verbana* would have ungulates as main host; however, direct evidence of such alleged preference is to date not available and in need of being corroborated. Lacking sufficient data, no sound conclusions can be currently draft on the diet of *Hirudo verbana*, although an opportunistic feeding behaviour of the species is rather likely. The realisation of analyses on the stomach content of wild individuals of the species is of paramount importance to better understand the trophic resources needed for the species in different sites and different seasons. Studying the dietary composition of a species in detail, in fact, is crucial to focus on its role in the trophic webs of the ecosystems it occupies, and to better understand the structure and trophic relationships of its ecosystem (Soulé et al., 2003). For this reason, understanding feeding habits also has an important function for the management of species, both if they have a dysfunctional position in ecosystems (e.g., invasive species, see Shik and Dussutour, 2020; Nogueira et al., 2023) and if they are poorly known, declining, or threatened taxa (Margalida et al., 2009; Ducotterd et al., 2020; Faraone et al., 2021). We hope that this report might foster the realisation of such studies.

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